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Gas Supersaturation Thresholds for Bubble Formation in and Damage to Sea Urchin Embryos

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Abstract. Eggs and early embryonic stages of the sea urchin *Lytechinus pictus* were subjected to hyperbaric gas pressures and rapid decompression. All stages showed a remarkable tolerance to gas supersaturations. No damage or internal bubbles were apparent in the eggs after decompression from less than 240 atm nitrogen or 209 atm argon. This indicates (1) a greater resistance to bubble formation than occurs in other invertebrates and vertebrates and (2) a lack of nucleation sites, such as hydrophobic interfaces in contact with the intracellular water. These thresholds decreased gradually to 170 atm and 148 atm, respectively, for 80-h-old plutei. Gas supersaturations above the threshold values often led to formation of internal bubbles, most frequently observed in the eggs. Slow decompression experiments usually had little effect on the organisms, showing that gas supersaturations were the cause of the damage rather than the hydrostatic pressures, the gases *per se*, or the hyperbaric conditions inherent in the procedures.

Introduction

In studies of bubble formation in cells subjected to hyperbaric gas pressures and subsequent decompression, it has been found that unicellular organisms such as *Tetrahymena, Euglena, Dictyostelium,* and bacteria as well as erythrocytes tolerate extreme gas supersaturations without bubbles forming internally (Hemmingsen and Hemmingsen, 1978, 1979, 1983; Hemmingsen *et al.,* 1985). Generally the cells are unaffected by nitrogen supersaturations up to 150 atm—and many even by supersaturations in excess of 200 atm—which is sufficient to cause profuse spontaneous formation of bubbles in water and aqueous solutions (Hemmingsen, 1977). Although *Euglena, Dictyostelium,* and *Tetrahymena* eventually show signs of damage (loss of colony-forming ability or decrease in number of cells), bubbles have been observed to form only in cells of *Tetrahymena.* These intracellular bubbles occur when the cells contain food vacuoles, which may act as nucleation sites, but only at gas supersaturations approaching the threshold for spontaneous nucleation in water (Hemmingsen, 1982; Hemmingsen and Hemmingsen, 1983).

Because the cell types that have been examined so far are relatively small (7000 μm^3 or less) and since the cell interior constitutes a somewhat isolated environment, it has been speculated (Hemmingsen *et al.,* 1985) that the underlying cause of the remarkable tolerance of cells to gas supersaturation is a lack of sufficient quantities of intracellular water. This view assumes that the spontaneous formation of a nucleus must be supported by a surrounding body of water of minimum size for a given content of dissolved gas.

This investigation was undertaken to obtain some evidence that the availability of water and cell size may be important factors affecting the nucleation of bubbles in cells. Eggs and early embryonic stages of the sea urchin *Lytechinus pictus* were selected for these experiments. The eggs are easily collected, fertilized and reared, and all stages up to metamorphosis are relatively transparent. Also, the eggs have a much larger volume (approximately 500,000 μm^3) than the cells used previously.

Other investigations into the effects of gas supersaturating conditions on fish and crustacean larvae have indicated that the younger organisms are more resistant to bubble formation (McDonough and Hemmingsen, 1984, 1985; Gray *et al.,* 1985). Our goal was to test sea

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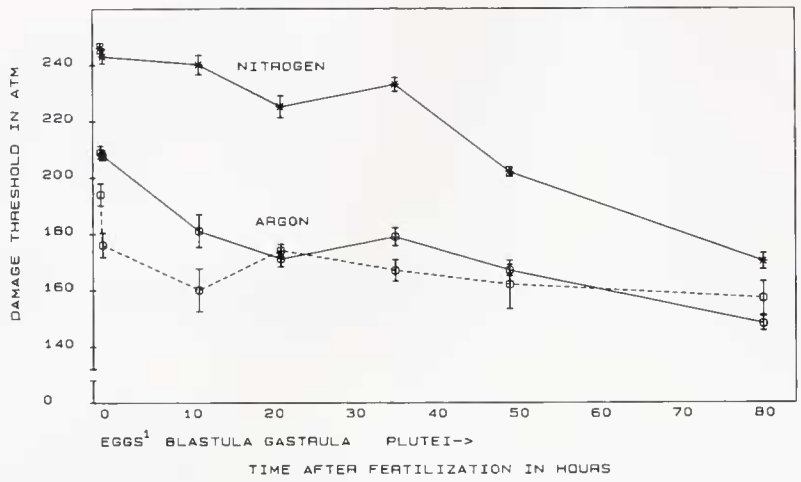

Figure 1. Mean gas supersaturation thresholds for damage to sea urchin eggs and early development stages. The means were computed using the midvalue of a range of pressures that bracketed the significant damage threshold. When the pressures tested did not provide values above and below the 40% significance level the pressure with damage closest to the significance level was used in computing the mean. Two methods of agitation were used during these experiments; shaken samples are represented by a solid line and stirred samples with a stippled line. Error bars represent the standard error. [1]The upper left values at time zero of each line represent unfertilized eggs while the values to the lower right, just off the zero of each line, represent eggs tested approximately 5 minutes after fertilization.

urchins' tolerance to gas supersaturation throughout the developmental process, and to attempt to correlate any changes in the damage threshold with morphological developments such as the subdivision of the cytoplasm or the formation of the blastocoel. Because early embryonic stages do not feed, the transition to a feeding larval stage enabled us to examine the impact of the ingestion of potential nucleating agents, such as gas nuclei or particles, on the supersaturation tolerances of the larvae.

Materials and Methods

The eggs of the sea urchin *Lytechinus pictus* were collected by intracoelomic injection of 0.5 *M* KCl, rinsed twice in seawater, and kept in a water bath at 16–18°C until used. Sperm were collected in a similar manner, concentrated, and stored on ice until used. Fertilized eggs and embryos were kept in a beaker of seawater at room temperature (21–25°C) (Hinegardner, 1975; Hinegardner and Rocha Tuzzi, 1981). Debris and dead embryos were removed daily from the beaker with a pipette; 10–20 ml fresh seawater was added. Unfertilized and fertilized eggs, blastula, gastrula, and pluteus stages of *L. pictus* were used in the experiments.

The threshold for damage and/or bubble formation was tested for each developmental stage by exposing 1 to 2 ml seawater containing the organisms to various gas saturations, followed by decompression. The experimental apparatus is described elsewhere (Hemmingsen and Hemmingsen, 1978, 1979). The small pressure chamber containing the sample was agitated by shaking (3 cycles/s) or by magnetic stir bar (*ca.* 200 rpm) during the gas exposure while a duplicate sample was simultaneously agitated in air at ambient pressure (1 atm). A 30-min equilibration time was used for most experiments; additional time for equilibration did not affect the threshold for damage, except for the unfertilized eggs subjected to shaking. In this latter case 1 to 2 hour equilibration times were used. After equilibration, the gas pressure in the chamber was rapidly released with complete decompression occurring within 2 s (fast decompression). In some experiments the gas was discharged slowly in steps at rates varying from 25 to 100 atm every 10 min (slow decompression). These slow decompression experiments were performed to reduce gas supersaturation, thereby separating its effects from others inherent in the procedure such as hydrostatic pressure, hypoxic conditions, and the gases themselves, all of which are potentially damaging.

At the beginning and end of each experiment on stages through the blastula, three subsamples of the stirred suspensions were collected in glass capillaries (75 mm × 1.1 or 1.5 mm) to count (at 75–150× in a compound microscope) the number of intact organisms per unit volume (Hinegardner, 1975) and to assess the degree of damage sustained. Six subsamples were used for the gastrula and later stages to compensate for the decreased number of

Table I

Comparison of bubble occurrence and frequency

Experimental conditions	Developmental stage	Total # experiments	Pressure range tested	# Experiments with internal bubbles[a]	Bubble occurrence pressure range	% Experiments with internal bubbles
Argon shaken	unfertilized eggs	60	175–220	19	210–220	32
	fertilized eggs	56	195–220	23	195–215	41
	blastulae	9	165–185	1	185	11
	gastrulae	17	165–195	4	165–185	24
	plutei	25	145–185	6	165–185	24
Nitrogen shaken	unfertilized eggs	13	230–250	1	240	8
	fertilized eggs	12	230–250	5	240–250	42
	blastulae	10	220–250	0	N/A	0
	gastrulae	17	200–240	1	230	6
	plutei	21	160–240	9	170–240	43
Argon stirred	unfertilized eggs	18	150–205	2	195–205	11
	fertilized eggs	8	155–185	0	N/A	0
	blastulae	8	145–175	1	165	13
	gastrulae	20	155–185	1	175	5
	plutei	46	145–185	13	155–185	28

[a] Defined as an experiment in which at least one organism was found to contain an internal bubble.

organisms per unit volume. Bubbles frequently formed in the capillary tubes filled with post-decompression subsamples. Repeated inversion of the capillary removed the bubbles and permitted a clear view of the contents and accurate determination of the volume. After the capillary subsamples had been removed, a few organisms were pipetted onto slides from the stirred suspensions and immediately observed. These observations were augmented by further examination of the capillary subsamples, which were first used for quantitative analysis then transferred to slides. This permitted examination of organisms and bubbles within 1 min of decompression and then 30–60 minutes later after the capillary contents had been counted.

An organism was considered damaged and therefore was not counted as having survived decompression if it exhibited any of the following characteristics: (1) obvious leakage of cytoplasm; (2) very dense or grainy appearance, especially in the early stages; (3) ragged or torn membranes or tissues, especially in the plutei; or (4) fragmentation. A "significant" level of damage was a 40% difference in the capillary counts following decompression [% difference = (# organisms reference − # organisms after)/# organisms reference × 100]. The reference value for the number of organisms in a sample used in this calculation represents a baseline value established from the arithmetic mean of a series of capillary counts made before the experiments from the stock culture and after decompression from the unpressurized control samples. The multiple samples taken from the stock culture during any series of experiments displayed no systematic variability. The level of significance is the point at which 40% or more of the total number of organisms were damaged according to the criteria given above. This 40% level also corresponded to the onset of severe damage.

One difficulty with the capillary counting method is that when small numbers of organisms were present, the method would occasionally (less than 10% of the time) yield a larger number of organisms after the experiment than before. When the percent difference numbers calculated from these values were required for additional calculations, they were set equal to zero because the negative values for the percent difference were obviously an artifact. Since this error appeared largely in experiments on small populations below threshold levels, it was rarely a factor in determining the damage threshold values.

Results

All developmental stages showed a remarkably high tolerance to gas supersaturation. The threshold for inducing damage in eggs is in excess of 240 atm nitrogen and 200 atm argon. Even larval plutei can tolerate more than 170 atm nitrogen and 140 atm argon. In most experiments performed above the threshold level more than one-third (range 10–100%) of the remaining organisms, apparently undamaged and often bubble-free, slowly disintegrated within 5 to 10 minutes following decompression. In these organisms which were intact after decompression, one or more bubbles were sometimes (in about 25% of the experiments) present. Bubbles

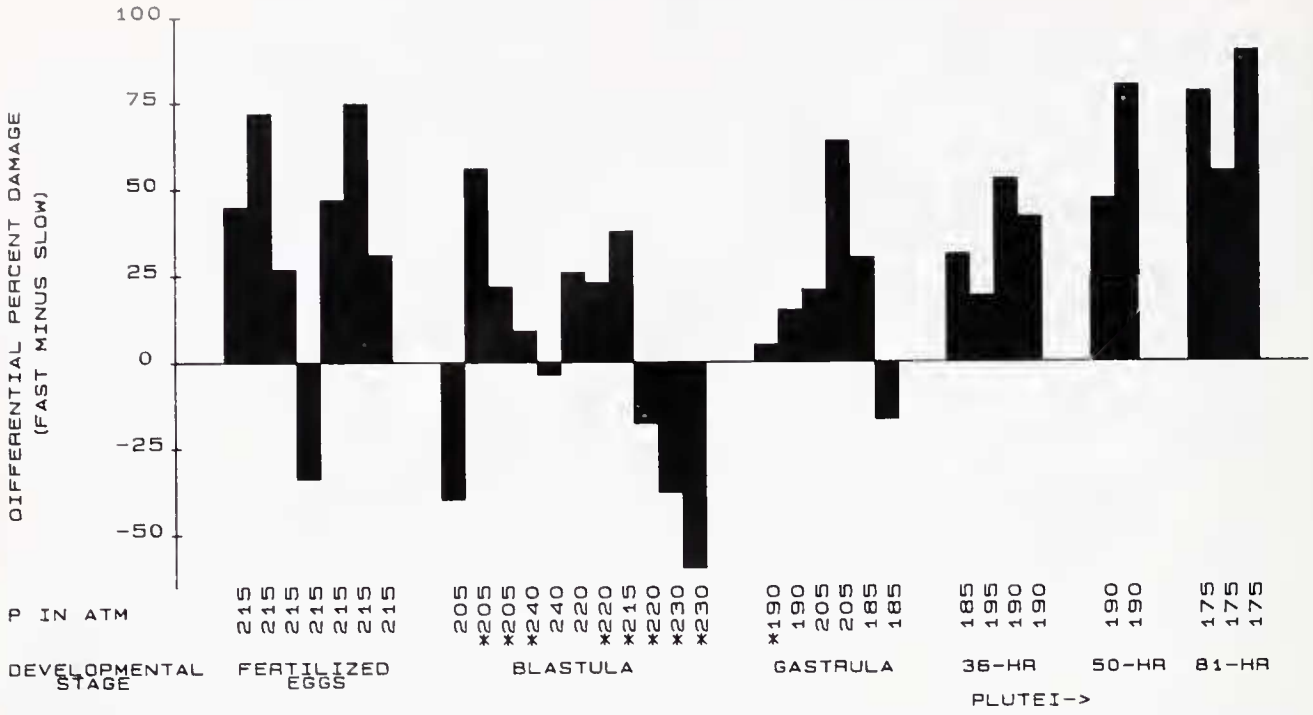

Figure 2. Comparison of damage inflicted on sea urchin eggs and early development stages by either fast or slow decompressions. The percent damage sustained during a fast decompression trial minus the percent damage of a slow decompression trial is plotted as the differential percent damage. A paired fast and slow decompression were performed on a sample from the same stock culture and equilibrated to the same final pressure. The decompression rate for the slow experiments was 50 atm every 10 minutes except for those bars marked with an asterisk (*) in which the slow decompression rate was 100 atm every 10 minutes.

were found in all developmental stages, but the frequency of their occurrence varied with the type of gas used, method of agitation, level of gas supersaturation, and stage of development (Table I). The minimum gas supersaturation required for bubble formation was 155 atm argon for plutei, but went as high as 250 atm nitrogen for fertilized eggs. Multiple internal bubbles were observed exclusively in eggs and then only when nitrogen was used; no more than three bubbles per egg were ever observed.

The level of gas supersaturation required for damage generally decreased as the embryos developed (Fig. 1). With either argon or nitrogen the threshold decreased as the eggs developed into the blastula or gastrula stage, then increased slightly before consistently decreasing through the pluteus stages. The overall damage threshold was 20 to 50 atm higher when nitrogen gas was used in place of argon.

Slow decompressions generally were less damaging to the organisms than fast decompressions (Fig. 2). With the egg, gastrula, and pluteus stages the fast decompressions damaged large numbers of organisms in at least 80% of the experiments performed. However, for the blastula stage only 45% of the fast decompression experiments resulted in more damage than the slow decompressions.

The thresholds for damage of the unfertilized and fertilized eggs were significantly ($P = .95$, two tailed t-test) affected by the method of agitation used; frequently samples equilibrated by stirring yielded a lower threshold for damage than those equilibrated by shaking agitation (Fig. 1). Since this difference could be associated with equilibration times, the effect of prolonged equilibration with argon on eggs was examined (Fig. 3). Extending the shaking agitation of fertilized eggs from 1 to 2 hours had little effect whereas unfertilized eggs sustained more damage. Because this effect had little bearing on our main objectives it was not explored further.

Discussion

The sea urchin eggs and early developmental stages all displayed striking tolerances to gas supersaturation, similar to those of many eucaryotic microorganisms, and

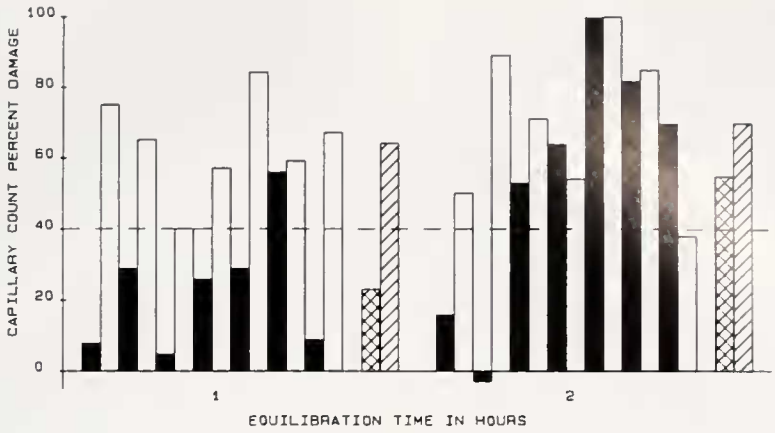

Figure 3. Damage to unfertilized and fertilized eggs following equilibration with argon at 210 atm for 1 and 2 hour with subsequent rapid decompression. Each bar represents the percent damage incurred by unfertilized eggs (solid bars) and fertilized eggs (open bars) in 14 separate trials. The mean values for damage sustained at each equilibration length are reported for unfertilized eggs (cross-hatched bars) and fertilized eggs (slashed bars).

much higher than those obtained for other invertebrates and vertebrates (Hemmingsen and Hemmingsen, 1979, 1983; McDonough and Hemmingsen, 1984, 1985). Also, in most cases the spread between supersaturations which produce slight damage and high survival and those which produce consistant damage and low survival is quite narrow (less than 10 atm). The resistance of the egg cells to bubble formation indicates (1) a lack of pools of free water of sufficient size to support spontaneous bubble nucleation in spite of the considerable size of the eggs (about 100 μm in diameter); (2) that a limited overall cell volume is not a prerequisite for obtaining the extraordinary resistance to bubbles that has been observed in most of the unicellular organisms studied so far; and (3) that the intracellular environment is void of hydrophobic interfaces in direct contact with the aqueous phase, since such interfaces are likely to destabilize the intracellular environment thereby creating conditions that favor nucleation.

In general there is a decrease in the threshold for damage during embryonic development. Internal bubbles developed in the eggs and in the other stages at supersaturations equal to or higher than the thresholds for damage (compare Fig. 1 and Table I); for the eggs and the blastulae, these gas supersaturations were substantially higher than those required for spontaneous nucleation of bubbles in water (Hemmingsen, 1977). The formation of intracellular bubbles in the eggs is an unusual phenomenon. Among the numerous types of cells studied, bubbles have previously been observed only in the ciliate *Tetrahymena* containing food vacuoles. The formation of bubbles in *Lytechinus* eggs requires gas supersaturations greater than those for spontaneous nucleation of bubbles in water (Hemmingsen, 1977); those in *Tetrahymena* form with gas supersaturation levels somewhat below the threshold for water, possibly because of particle surfaces within the food vacuole (Hemmingsen and Hemmingsen, 1983).

The presence of the fluid-filled blastocoel was expected to cause the blastulae to have a damage threshold more similar to that of bulk water, since the cavity contains a relatively homogenous liquid lacking obvious macroscopic structure (Stearns, 1974). This fluid could then act as a pool of "free" water with normal nucleation properties, however this characteristic was not readily apparent. The blastulae actually exhibited the lowest mean frequency of internal bubbles and had a higher threshold for damage than most of the subsequent developmental stages (Fig. 1, Table I). The large standard error and variability (Figs. 1, 2) of the values associated with this stage made finer distinctions difficult.

The gastrula is a stage in rapid transition that appears to be slightly more susceptible to damage by gas supersaturation than the stages preceding or immediately following. It is unlikely that the damage threshold of this stage is dependent on the presence of skeletal rudiments (spicules) as they represent an incomplete framework and are rarely seen protruding from the organisms following decompression. The formation of the alimentary canal at this stage involves the inward migration of cells and the movement and attachment of cells via pseudopodia (Giudice, 1973). Although the introduction of these structures divides the internal fluid into smaller entities which may be more stable, it is also possible that the restructuring of this internal environment introduces new, destabilizing interfaces that may enhance nucleation.

The transition to a feeding larva is one of the major changes that occurs during the pluteus stage. When the plutei begin feeding, ingestion of gas micronuclei and particulate substances from the seawater could promote bubbles in the digestive tract which upon expansion would damage the organisms. Indeed, many of the bubbles observed in the plutei were in the region of the mouth, and some otherwise intact plutei had a damaged alimentary canal. The presence of a complete internal skeleton may also be important in determining the damage threshold for this stage. Slight protrusion of the skeleton is minimally important to the long-term survival of the plutei following decompression because repair occurred within 24 hours. Extrusion of the skeleton became more critical as we approached the damage threshold for the plutei and the numbers of internal bubbles increased. This increased the stretching of the tissue and the likelihood that the skeleton would puncture and rip it at stress points. It is thus in relation to the rigid framework provided by the skeleton and the stretching imposed on the tissues by internal bubbles that the presence of a skeletal framework may affect the threshold for damage.

Overall, the fast and slow decompression experiments conducted on the plutei were more consistent than for other stages. This might partially reflect the fact that the initially heterogeneous group of embryos were refined by the stressful conditions of the culture so that only the strongest and healthiest survived to the pluteus stage. Also, this stage has a skeleton and other internal structures that may help to stabilize it against the impact of both external and internal bubbles.

The damage observed in all stages was largely caused by the gas supersaturation produced by rapid decompression, since the slow decompressions, which eliminated most of the gas supersaturation and related bubble formation, generally resulted in less damage (Fig. 2). The exception is the blastula stage (Fig. 2) for which the prolonged exposure necessary for the slow decompressions appeared to have a detrimental effect. The slow decompression data for all other stages show that they are not much affected by the gas exposure *per se*, the hydrostatic pressure, or the hypoxic conditions inherent in the experimental procedure.

Whereas fast decompression from threshold levels or greater yielded debris and large pieces of tissue from organisms that had ruptured during decompression, slow decompressions from the same levels resulted only in organisms that were grainy and wrinkled but otherwise undamaged; little debris was evident. This suggests that the damage suffered during the slow decompression is largely due to factors intrinsic to the procedure, such as the length of gas exposure, rather than to bubble formation. The slow decompression experiments were not conducted on the unfertilized eggs because the duration and number of experiments required would have reduced the integrity of these eggs, as they usually begin to disintegrate a few hours after spawning even at atmospheric pressure.

The significant threshold differences observed for the early stages when comparing the two methods of agitation (Fig. 1, Table 1) may be the result of a higher sensitivity of these stages to the shear forces present when stirring. For the egg stages though, these threshold differences extend to shaking experiments when shorter equilibration times were compared with those longer than 1 hour. The cause of these time-dependent threshold differences has not been determined. Some evidence suggests that there are different permeabilities for the membranes of unfertilized and fertilized *Lytechinus* eggs with respect to gases since permeability differences have been reported for other substances such as water and ethylene glycol (Lillie, 1916; Stewart and Jacobs, 1932). However, recent investigations (Merta *et al.*, 1986) suggest that differences such as these may actually depend on the character and quantity of the intracellular water present in unfertilized *versus* fertilized eggs. Further studies are required to resolve this question of equilibration differences.

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Literature Cited

Giudice, G. 1973. *Developmental Biology of the Sea Urchin Embryo.* Academic Press, New York. Pp. 14–18.

Gray, R. H., M. G. Saroglia, and G. Scarano. 1985. Comparative tolerance to gas supersaturated water of two marine fishes, *Dicentrarchus labrax* and *Mugil cephalus*. *Aquaculture* **48:** 83–89.

Hemmingsen, B. B., and E. A. Hemmingsen. 1978. Tolerance of bacteria to extreme gas supersaturations. *Biochem. Biophys. Res. Commun.* **85:** 1379–1384.

Hemmingsen, B. B., and E. A. Hemmingsen. 1983. Intracellular bubble formation: difference in gas supersaturation tolerances between *Tetrahymena* and *Euglena*. *J. Protozool.* **30**(3): 608–612.

Hemmingsen, B. B., N. A. Steinberg, and E. A. Hemmingsen. 1985. Intracellular gas supersaturation tolerances of erythrocytes and resealed ghosts. *Biophys. J.* **47:** 491–496.

Hemmingsen, E. A. 1977. Spontaneous formation of bubbles in gas-supersaturated water. *Nature* **267:** 141–142.

Hemmingsen, E. A. 1982. Cinephotomicrographic observations on intracellular bubble formation in *Tetrahymena*. *J. Exp. Zool.* **220:** 43–48.

Hemmingsen, E. A., and B. B. Hemmingsen. 1979. Lack of intracellular bubble formation in microorganisms at very high gas supersaturations. *J. Appl. Physiol. Respir. Environ. Excercise Physiol.* **47**(6): 1270–1277.

Hinegardner, R. 1975. Care and handling of sea urchin eggs, embryos, and adults (principally North American species). Pp. 15 in *The Sea Urchin Embryo: Biochemistry and Morphogenesis,* G. Czihak, ed. Springer-Verlag, Berlin, Heidelberg, New York.

Hinegardner, R. T., and M. M. Rocha Tuzzi. 1981. Laboratory culture of the sea urchin *Lytechinus pictus.* Pp. 291–304 in *Laboratory Animal Management: Marine Invertebrates,* National Research Council (U.S.) Committee on Marine Invertebrates. National Academy Press, Washington, DC.

Lillie, F. R. 1916. Increase of permeability to water following normal and artificial activation in sea urchin eggs. *Am. J. Physiol.* **40**(2): 249–266.

Merta, P. J., G. D. Fullerton, and I. L. Cameron. 1986. Characterization of water in unfertilized and fertilized sea urchin eggs. *J. Cell. Physiol.* **127**: 439–447.

McDonough, P. M., and E. A. Hemmingsen. 1984. Bubble formation in crustaceans following decompression from hyperbaric gas exposures. *J. Appl. Physiol. Respir. Environ. Exercise Physiol.* **56**(2): 513–519.

McDonough, P. M., and E. A. Hemmingsen. 1985. Swimming movements initiate bubble formation in fish decompressed from elevated gas pressures. *Comp. Biochem. Physiol.* **81A**(1): 209–212.

Stearns, L. W. 1974. *Sea Urchin Development: Cellular and Molecular Aspects.* Dowden, Hutchinson, and Ross, Inc., Stroudsburg, PA. Pp. 115–118.

Stewart, D. R., and M. H. Jacobs. 1932. The effect of fertilization on the permeability of the eggs of Arabacia and Asterias to ethylene glycol. *J. Cell. Comp. Physiol.* **1**: 83–92.